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DIFFERENT MICE INBRED STRAINS HUMORAL IMMUNE RESPONSE TO HORSERADISH PEROXIDASE

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Introduction

Peroxidases belonging to the class of oxidoreductases, catalyze the oxidation of a wide range of substrates under the influence of hydrogen peroxide. They are divided into two major superfamilies: animal and plant enzymes. Plant peroxidases include three classes [1]. Isoenzyme C of horseradish peroxidase from roots (horseradish peroxidase, HRP) (EC 1.11.1.7) is the most researched and used among all peroxidases and is used as a model for the third class of plant peroxidases. HRP is a glycoprotein containing prosthetic group (Fe(III)-protoporferyn-IX), 308 amino acid residues and 8 oligosaccharide chains [2]. The molecular mass (MM) of the polypeptide chain is 33,899 Da, and glycosylated protein is about 44 kDa. HRP molecule contains 4 disulfide bonds and two calcium ions. The main feature of this molecule is the presence of two domains and gem localised in the cavity between them [2]. Isoelectric point (PI) of HRP is 9.0. Enzyme's biological activity maximum is shown at pH 6.8 [1]. HRP is used as a marker in immunological tests, biosensors, gene therapy, histological studies and as catalyser in bio-organic synthesis [1, 3-6]. Such activities sometimes need use of bioreagents – specific antibodies to HRP. The most appropriate is specific monoclonal antibodies (mAbs), as they, unlike polyclonal antibodies are characterized by exceptional specificity, homogeneity and possibility of obtaining in unlimited quantities. Constant identity of mAb isotype and idiotype from batch to batch ensures their constant appropriate quality and hence the reproducibility of analytical methods [7]. One of the important elements of the hybridoma technology is the use of effective immunization schemes that would provide an extensive epitop repertoire of mAb with high affinity and activity in different immunoassay methods.

The aim of the study has been investigation of humoral immune response to horseradish peroxidase of different mice inbred strains for further development of recommendations for appropriate immunization schemes for monoclonal antibodies obtaining.

Materials and methods

Animals. Balb/c and NZB mice were used for immunization: females aged 6-8 months, 20-22 g weight. The animals were kept in cages in natural light, temperature 20-22 °C; access to food and water was free. Studies on animals were carried out in compliance with bioethical norms [8]. All the work has been used 72 animals.

Immunogen. Horseradish peroxidase type I (purchased by Sigma, USA) used as immunogen was a demineralized freeze-dried substance with a specific activity of 50-150 units/mg (using pyrogallol as substrate). In case of booster immunization immunogen was previously diluted to the desired concentration (10, 25, or 50 mcg per 100 mcl) in saline and injected in the tail vein. In case of other immunizations we prepared emulsion solution of immunogen with adjuvant to final concentration of 10, 25, or 50 mcg per 100 mcl: HRP was dissolved in saline, the same volume of adjuvant was added, and mixture was thoroughly mixed to form a stable emulsion. When subcutaneous administration total dose of 100 mcl was divided into two equal parts and was injected in the hind paw of mice. Intraperitoneal immunization was performed by single administration of 100 mcl of emulsion. Immunogen solution (emulsion) was prepared immediately before immunization.

Procedure of conjugate of polyclonal antibodies and alkaline phosphatase (ALP) obtaining. 3 mg of ALP with activity of 1000 units/mg (Sigma, USA) was dissolved in 100 ml of 0.1 phosphate buffer, pH 6.8. 300 ml (concentration 10 mg/ml) of solution of purified goat antibodies against mouse IgG was added. Glutaraldehyde was added to the mixture up to the final concentration of 1% and

mixture was incubated for 3 h at 20 °C. Reactive mixture was dialyzed overnight at 4 °C against phosphate buffer with 0.05 M Tris-HCl, 0,5 M NaCl and 1 mM $MgCl_2$ (pH 8.8) [9]. The activity of the resulting conjugate was 600-700 units/mg (determined by the method [10]).

Enzyme-linked immunosorbent assay (ELISA). We used two versions of ELISA (direct and indirect analysis) to detect specific anti-HRP antibody in the blood serum of experimental animals. Direct ELISA was performed as follows. Protein A of *Staphylococcus aureus* (4 mg/ml in 0.02 M carbonate buffer (pH 9.5)) was immobilized on the surface of the polyester plates with high sorption capacity (Suzhou Conrem Biomedical Technology Co., China) overnight at 4 °C (100 mcg/well). After washing the plates by phosphate saline buffer with 0.05% Tween-20, pH 7.2-7.4 (FSBT) titration of mice blood sera (100 ml/well) was conducted. After incubation for 1 h at 37 °C and washing with FSBT HRP-specific antibodies detection was carried out with peroxidase solution (10^{-7} M) in FSBT (100 ml/well). Plates were incubated 1 h at 37 °C, washed with FSBT. Enzyme activity was determined by adding of chromogenic and substrate solutions (3,3',5,5'-tetrametylbenzydyn, 0.003% H_2O_2, 0.15 M citrate buffer, pH 5.0). Optical density was measured at 450 nm (after enzymatic reaction stopping).

Indirect ELISA was performed as follows. HRP (5 mg/ml) was immobilized on the surface of the polyester plates with high sorption capacity and then samples of mice blood sera were incubated (for 1 h at 37 °C). After wells washing formed immune complexes were determined by incubation with conjugate of goat polyclonal antibody to mouse IgG with ALP. After preparing substrate and chromogenic mixtures (1 mg/ml p-nitrophenyl phosphate with 1 M diethanolamine and 0.5 M $MgCl_2$, pH 9.8). Optical density was measured at 450 nm (after enzymatic reaction stopping). As a control in both versions of ELISA we used the unimmunized mice serum.

Results and discussion

Rationale immunization schemes. The prerequisites for comparative studies of different immunization schemes effectiveness for obtaining antibodies to HRP were the results of previous experiments with obtaining of mAb to HRP using the following scheme of immunization.

Immunization of Balb/c mice was performed subcutaneously (s/c) in the hind paw of mice quote: the first two injections were performed with complete Freund's adjuvant (CFA) (Sigma, USA), and the third – without adjuvant; three months after the third injection booster dose of HRP was injected intravenously (i/v) into the tail vein. On the third day hybridization of splenocytes from mice spleen cells and Sp 2/0 myeloma was performed. The total dose of immunogen was 75 mg of HRP per mouse.

When using such a scheme as a result of immunization hybridization manifested significantly less positive clones hybrid than usual [11, 12]: after initial testing only in a quarter of the wells culture plates were found positive clones. Monoclonal antibodies, which were synthesized by obtained hybridomas, were characterized rather low titer (less then 1:5,000) and affinity constant (less then 10^{-8} M^{-1}). It should also be noted that the HRP enzyme as immunogen was quite toxic: inflammatory response up to the partial necrosis of pads rear legs was observed in experimental animals paws. Thus, horseradish peroxidase is weak and toxic to animals immunogen. Therefore, we were forced to develop such a scheme of immunization which could neutralize abovementioned negative factors.

Results of our previous experiments correlate with data of other authors. For example, in work [13] were presented the results of obtaining of anti-HRP mAbs using Balb/c mice according to the following immunization scheme: three consecutive intraperitoneal (i/p) immunizations (2 mg per administration) with an interval of 30 days and a booster intravenous immunization (5 mg). As a result of 7 hybridizations using described immunization scheme efficiency of positive hybrid clones formation was extremely low (0 to 5.3 %), and obtained mAbs had low activity in ELISA. Other authors [14] have reported successful results of rat mAbs obtaining using relatively high immunogen doses (two successive i/p injections of Lou/m rats at 500 mg HRP and booster administration in the same amount of HRP). Although in this study yield of hybridomas positive clones was also low.

In some studies [15, 16] were described the immunization scheme for so-called “weak” immunogens. In our opinion, experimental works [17-19] using mice NZB, which are characterized by a high level of B-cell immune response, are of special interest. The abovementioned facts led us to a

series of experiments to establish the level of humoral response of mice of different lines (Balb/c, and NZB) with multi immunization, different duration, varying amounts of HRP (10, 25, and 50 mg), with different route of immunogen administration (i/p, and s/c). Generalized scheme of the experiment is shown in fig. 1.

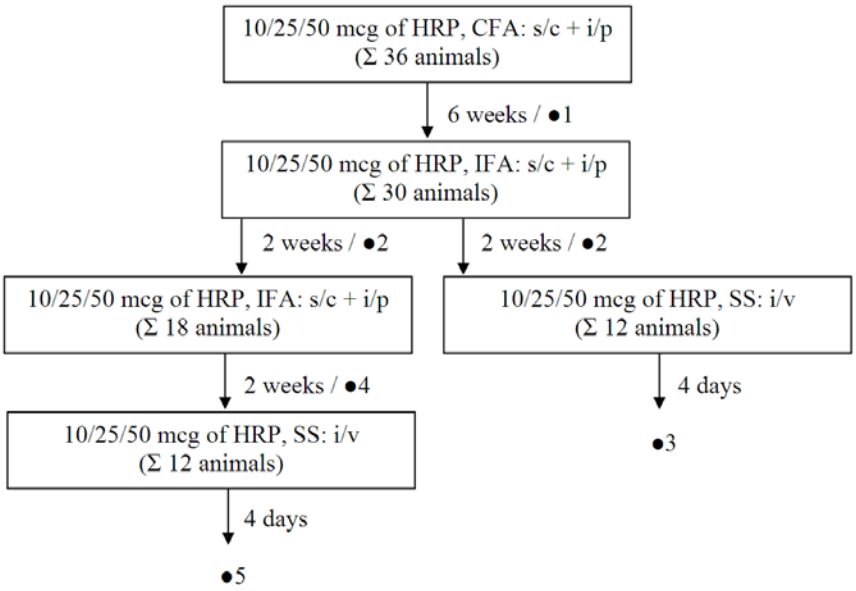

Figure 1 – Schemes for Balb/c and NZB mice immunization by horseradish peroxidase (CFA – complete Freund's adjuvant; IFA – incomplete Freund's adjuvant; SS – saline solution; ● – points for blood sampling for the study of specific antibodies titer)

Comparison of different immunization schemes of Balb/c and NZB mice. To assess the level of humoral immune response to HRP it was necessary to evaluate the applicability of various ELISA modifications (direct and indirect analysis) to detect specific antibodies in the serum of animals. A comparative study of the titration of sera in the direct and indirect ELISA showed comparable results, so further studies were performed using a direct ELISA, which, first, is more easy to perform, and, secondly, does not require expensive biological reagents (conjugates based on ALP).

The evaluation of the data on the dynamics of the humoral immune response of mice of different lines with different immunization schemes are presented in fig. 2.

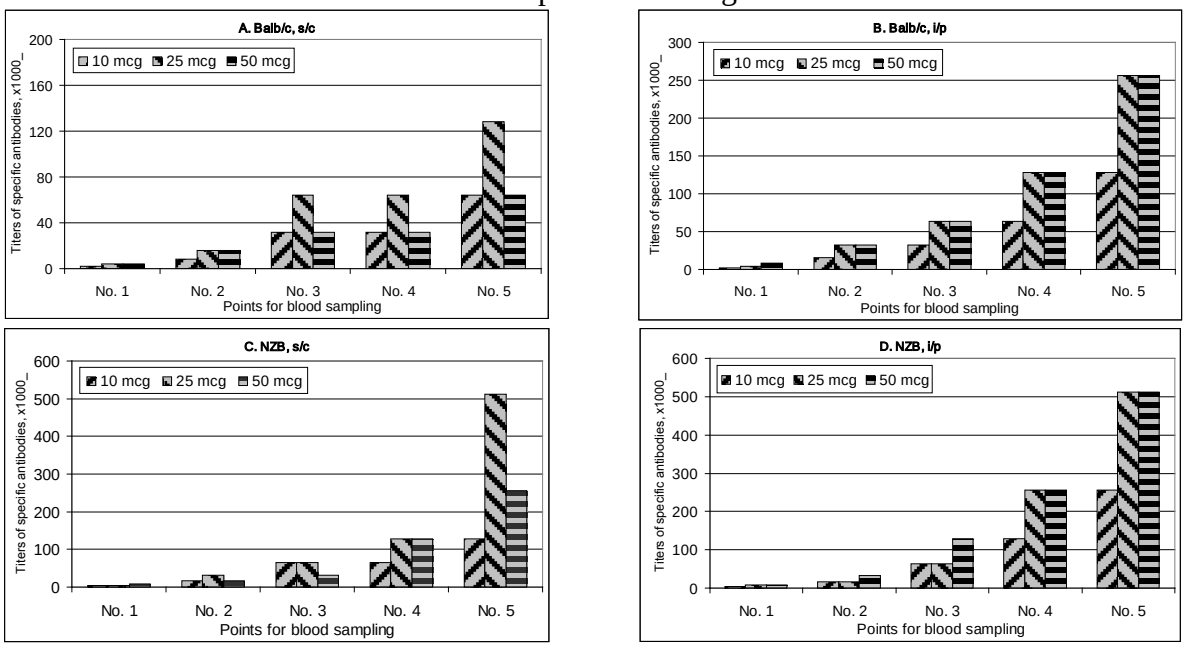

Figure 2 – Results of determination of specific antibodies in the serum of mice at different immunization schemes

Mice repeated immunizations expectedly increased level of humoral immune response against HRP. However i/p immunization provided more intensive growth of specific antibodies levels in the serum of both strains of mice comparably with the same s/c immunization (fig. 2 A and Fig. 2 C compared with Fig. 2 B and Fig. 2 D, respectively). Especially, this difference is noticeable when testing sera in experimental points no. 4 and no. 5. More pronounced immune response in conducting a series of experiments were recorded for mice NZB: specific antibody titers were higher as for s/c immunization, and for i/p immunogen administration.

Interesting results were obtained at determination of the dependence of the level of humoral immune response from immunogen dose. The highest dose of HRP (50 mcg) in the case of c/s administration did not cause an increase in titer of specific antibodies compared with lower doses of immunogen: i/p immunization of 50 mcg of HRP ensured the formation of the same antibody titer for both doses of 25 mcg or even lower titer (notably in the points of the experiment no. 3÷5). In the case of i/p HRP immunization we did not observe such pattern at the highest dose administered immunogen. These results can be explained by inflammatory and degenerative processes in the hind limbs of mice with s/c administered immunogen, which increased during the experiment. Obviously, the effectiveness depends on the effectiveness of immunogen delivery to immune cells. With increasing degenerative processes in the hind legs of animals, obviously deteriorated HRP delivery to spleen cells via the bloodstream. Thus, the most active immune response to HRP was detected in mice NZB. At the same dose of 25 mcg has provided the highest level of specific antibodies (fig. 2 D). In the experiments, we injected booster dose of immunogen after two (8 weeks) and three (10 weeks) consecutive immunizations (control point no. 3 and no. 5). It was shown that a 10-week immunization scheme provides formation significantly higher antibody titers in the serum of animals.

Conclusion

Dependencies of the humoral immune response of Balb/c and NZB mice against horseradish peroxidase on the route of administration of immunogen, the dose and duration of immunization were established. It was shown that i/p administration provided formation of higher titers of specific antibodies in case of both mice strains. NZB mice lines more rapidly responded to HRP, than a Balb/c mice (for all investigated schemes immunization). It was shown that the most effective immunization scheme was three times i/p administration with 25 mcg of HRP for 10 weeks (the first immunization with CFA, and the rest with IFA) and booster immunogen i/v administration in saline solution.

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